

[0284] Four targets were chosen for this experiment: targets 2, 10, 14 and 16 (SEQ ID NO: 168, 188, 198 and 220, respectively). GFP fragment dsRNA (SEQ ID NO: 235) was used as a control. Young adults (2 to 3 days old) were picked at random from our laboratory-reared culture with no bias towards insect gender. Ten adults were chosen per treatment. The adults were prestarved for at least 6 hours before the onset of the treatment. On the first day of treatment, each adult was fed four potato leaf discs (diameter 1.5 cm²) which were pretreated with a topical application of 25 µl of 0.1 µg/µl target dsRNA (synthesized as described in Example 3A; topical application as described in Example 3E) per disc. Each adult was confined to a small petridish (diameter 3 cm) in order to make sure that all insects have ingested equal amounts of food and thus received equal doses of dsRNA. The following day, each adult was again fed four treated leaf discs as described above. On the third day, all ten adults per treatment were collected and placed together in a cage consisting of a plastic box (dimensions 30 cm×20 cm×15 cm) with a fine nylon mesh built into the lid to provide good aeration. Inside the box, some moistened filter paper was placed in the base. Some (untreated) potato foliage was placed on top of the paper to maintain the adults during the experiment. From day 5, regular assessments were carried out to count the number of dead, alive (mobile) and moribund insects. For insect moribundity, adults were laid on their backs to check whether they could right themselves within several minutes; an insect was considered moribund only if it was not able to turn onto its front.

[0285] Clear specific toxic effects of double-stranded RNA corresponding to different targets towards adults of the Colorado potato beetle, *Leptinotarsa decemlineata*, were demonstrated in this experiment (FIG. 12-LD). Double-stranded RNA corresponding to a gfp fragment showed no toxicity towards CPB adults on the day of the final assessment (day 19). This experiment clearly showed that the survival of CPB adults was severely reduced only after a few days of exposure to dsRNA when delivered orally. For example, for target 10, on day 5, 5 out of 10 adults were moribund (sick and slow moving); on day 6, 4 out of 10 adults were dead with three of the survivors moribund; on day 9 all adults were observed dead.

[0286] As a consequence of this experiment, the application of target double-stranded RNAs against insect pests may be broadened to include the two life stages of an insect pest (i.e. larvae and adults) which could cause extensive crop damage, as is the case with the Colorado potato beetle.

Example 4

Phaedon cochleariae (Mustard Leaf Beetle)

A. Cloning of a Partial Sequence of the *Phaedon cochleariae* (Mustard Leaf Beetle) PC001, PC003, PC005, PC010, PC014, PC016 and PC027 Genes Via Family PCR

[0287] High quality, intact RNA was isolated from the third larval stage of *Phaedon cochleariae* (mustard leaf beetle; source: Dr. Caroline Muller, Julius-von-Sachs-Institute for Biosciences, Chemical Ecology Group, University of Wuerzburg, Julius-von-Sachs-Platz 3, D-97082 Wuerzburg, Germany) using TRIzol Reagent (Cat. Nr. 15596-026/15596-018, Invitrogen, Rockville, Md., USA) following the manufacturer's instructions. Genomic DNA present in the RNA

preparation was removed by DNase (Cat. Nr. 1700, Promega) treatment following the manufacturer's instructions. cDNA was generated using a commercially available kit (Super-Script™ III Reverse Transcriptase, Cat. Nr. 18080044, Invitrogen, Rockville, Md., USA) following the manufacturer's instructions.

[0288] To isolate cDNA sequences comprising a portion of the PC001, PC003, PC005, PC010, PC014, PC016 and PC027 genes, a series of PCR reactions with degenerate primers were performed using Amplitaq Gold (Cat. Nr. N8080240, Applied Biosystems) following the manufacturer's instructions.

[0289] The sequences of the degenerate primers used for amplification of each of the genes are given in Table 2-PC. Table 2-PC displays *Phaedon cochleariae* target genes including primer sequences and cDNA sequences obtained. These primers were used in respective PCR reactions with the following conditions: 10 minutes at 95° C., followed by 40 cycles of 30 seconds at 95° C., 1 minute at 55° C. and 1 minute at 72° C., followed by 10 minutes at 72° C. The resulting PCR fragments were analyzed on agarose gel, purified (QIAquick Gel Extraction kit, Cat. Nr. 28706, Qiagen), cloned into the pCR4/TOPO vector (Cat. Nr. K4530-20, Invitrogen) and sequenced. The sequences of the resulting PCR products are represented by the respective SEQ ID NO:s as given in Table 2-PC and are referred to as the partial sequences.

[0290] The corresponding partial amino acid sequence are represented by the respective SEQ ID NO:s as given in Table 3-PC. Table 3-PC provides amino acid sequences of cDNA clones, and the start of the reading frame is indicated in brackets.

B. dsRNA Production of the *Phaedon cochleariae* Genes

[0291] dsRNA was synthesized in milligram amounts using the commercially available kit T7 Ribomax™ Express RNAi System (Cat. Nr. P1700, Promega). First two separate single 5' T7 RNA polymerase promoter templates were generated in two separate PCR reactions, each reaction containing the target sequence in a different orientation relative to the T7 promoter.

[0292] For each of the target genes, the sense T7 template was generated using specific T7 forward and specific reverse primers. The sequences of the respective primers for amplifying the sense template for each of the target genes are given in Table 8-PC. Table 8-PC provides details for preparing ds RNA fragments of *Phaedon cochleariae* target sequences, including primer sequences.

[0293] The conditions in the PCR reactions were as follows: 1 minute at 95° C., followed by 20 cycles of 30 seconds at 95° C., 30 seconds at 60° C. and 1 minute at 72° C., followed by 15 cycles of 30 seconds at 95° C., 30 seconds at 50° C. and 1 minute at 72° C. followed by 10 minutes at 72° C. The anti-sense T7 template was generated using specific forward and specific T7 reverse primers in a PCR reaction with the same conditions as described above. The sequences of the respective primers for amplifying the anti-sense template for each of the target genes are given in Table 8-PC. The resulting PCR products were analyzed on agarose gel and purified by PCR purification kit (Qiaquick PCR Purification Kit, Cat. Nr. 28106, Qiagen) and NaClO₄ precipitation. The generated T7 forward and reverse templates were mixed to be transcribed and the resulting RNA strands were annealed, DNase and RNase treated, and purified by sodium acetate,